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Chapter 7

ENZYMATIC ALTERATION OF INSECT PHEROMONES

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". . . Now in these moths the antennae of the males are highly developed. It seems undoubtedly to be the case that since the males could not see the females, they discerned them through the sense of smell and this is further evidenced by the antennae, the seat of smell, being more fully developed in the males than in the females."
Samuel Hunter (1902) in "Elementary Studies in Insect Life"

1. INTRODUCTION

1.1. *Generalized insect olfactory system*

Insects perceive olfactory stimulants such as sex pheromones via chemosensory sensilla located primarily on the antenna. The cuticular surface of the olfactory sensillum is penetrated by pores that connect the external environment of the insect with the dendritic nerve endings (Figure 1A). In a typical lepidopteran olfactory sensillum, the pores open into a pore canal. From the pore canal a number of pore tubules extend into an extra-

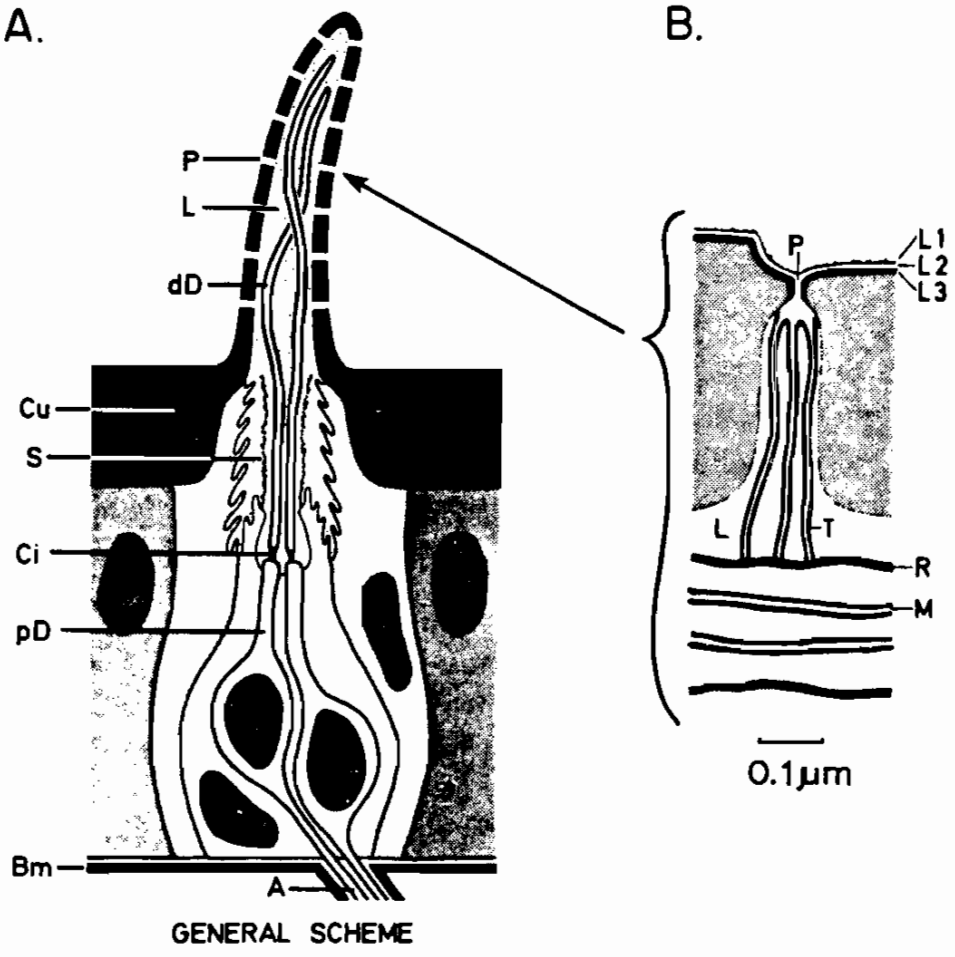

Figure 1. Schematic diagram of insect olfactory sensillum. A. General scheme of the insect olfactory sensillum in a longitudinal section. The receptor cells (white) are concentrically sheathed by the trichogen cell (light gray) and the tormogen cell (medium gray); unmodified epidermal cells are dark gray. B. Scheme of a single pore system and the layers of surface coat in males of *Bombyx mori* (from Steinbrecht and Kasang, 1972). A, axon; Bm, basal membrane; Ci, ciliary segment of the dendrite; Cu, cuticle; dD and pD, distal and proximal segments of dendrite; dM, dense material; L, sensillum liquor; L1, teuous membrane (approximately 75 A) that fills lumen of tubules; L3, thick, dense layer (approximately 85 A) that outlines the pore funnels and pore canals; M, cytoplasmic microtubule; P, pore system consisting of pore channel, pore kettle and several pore tubules (simplified in A); R, dendritic membrane; S, dendrite sheath; T, pore tubule (from Steinbrecht, 1969; Steinbrecht and Kasang, 1972).

cellular fluid called the 'sensillum liquor' and end at the dendritic membrane (Figure 1B). The pores and pore tubules may conduct the odorant by surface diffusion to acceptor sites located on the surface of the membrane (Kaissling, 1969; Kafka, 1971; Steinbrecht and Kasang, 1972; Norris, 1979). However, other possibilities for the function of the pores and pore tubules in transduction have been suggested (Zacharuk, 1971; Seabrook, 1977).

A generalized scheme for perception of insect pheromones has been presented by Kasang and Kaissling (1972) and Kasang (1973). According to Kaissling (1974) transduction of the pheromone stimulus into a receptor potential must involve at least six steps: (1) adsorption onto the antennal surface; (2) diffusion to acceptor sites; (3) binding; (4) activation (e.g., a conformation change) of acceptors; (5) change of membrane conductance; and (6) early inactivation of the pheromone molecule. Adsorption and diffusion are considered to be rather unspecific processes compared with the other steps which are compound-specific and contribute to the chemical specificity of the cell response.

1.2. *Significance of pheromone catabolism in perception*

Research on the overall process of pheromone perception in insects has primarily been concerned with the behavioral and neurophysiological responses to the odorant. Pheromone catabolism has received little attention. Degradation of insect pheromones on and/or in the antennae has been reported in the adult silkworm, *Bombyx mori* (L.) (transformation of (*E, Z*)-10,12-hexadecadien-1-ol to fatty acids, esters and alcohols (Kasang, 1971, 1973, 1974)), the gypsy moth, *Lymantria dispar* (L.) (conversion of *cis*-7,8-epoxy-2-methyloctadecane to two unidentified polar metabolites (Kasang et al., 1974a)) and the cabbage looper, *Trichoplusia ni* (Hübner) (hydrolysis of (*Z*)-7-dodecen-1-ol acetate to alcohol and acid metabolites (Ferkovich et al., 1972; Mayer, 1975)).

The significance of enzymes in the degradation of insect pheromones was first proposed by Schneider (1970). Pheromone degradation could play two roles, one in olfaction in the antenna and one in clearing the body surfaces of pheromone. Enzymes in the antenna might function in the olfactory process of the insect by serving to clear the pheromone from the receptor membrane subsequent to stimulation (Kasang, 1971). Kaissling (1972) proposed an early and a late inactivation of the pheromone molecule to explain several properties of the electroantennogram (EAG) response to bombykol in *B. mori*. Early inactivation would physically or chemically reduce the pheromone concentration at the acceptors within seconds and is primarily based on the immediate decay of the EAG after stimulation. For example, the odor molecule might be translocated into the sensillum liquor or to the inside of the dendrites after stimulation. Enzymatic degradation of radioactively labeled bombykol is apparently too slow (50% in 4 min) to account for the rapid response time of the EAG (Kasang and Kaissling, 1972). Late inactivation theoretically would remove the pheromone molecule from the sensory hairs over a matter of minutes and is based on penetration and catabolism of radioactively labeled bombykol on the antennae. Bombykol and two structural analogs, dihydrobombykol and

tetrahydrobombykol, were all degraded similarly, indicating a lack of enzymatic specificity. A similar lack of specificity was observed in enzymatic degradation of the pheromone of *L. dispar*, *cis*-7,8-epoxy-2-methyloctadecane, and its precursor, (*Z*)-2-methyl-7-octadecene (Kasang et al., 1974a).

Regarding cuticular enzymes, Kasang (1971) reported that the cuticle of the antenna and other body parts of *B. mori* were capable of transforming the pheromone into acid and ester metabolites. The observed catabolism was proposed to function in removing the absorbed pheromone from the bodies of both sexes which would otherwise later desorb, causing males to erroneously attract other males or females to attract males when they are no longer receptive to mating.

In my laboratory attention has been focused on pheromone catabolism in the cabbage looper moth, *T. ni*. Enzymatic hydrolysis of the pheromone in *T. ni* was first demonstrated in homogenates of the antennae and two other tissues. Pheromone degradation was more rapid in the antennal homogenate than in homogenates of the other tissues (Ferkovich et al., 1973). Studies in vivo demonstrated a specificity for pheromone when compared with six isomers and analogs on the antennae and legs of *T. ni*. Moreover, male antennae hydrolyzed the pheromone at twice the rate of female antennae (Mayer, 1975). When a sonication technique was developed to isolate the 'sensillum liquor' and membranes from the antennal chemoreceptor sensilla, pheromone-degrading esterases were found in the soluble protein fraction and were also associated with membrane vesicle fraction (Mayer and Ferkovich, 1976). These results suggested that the antennae contained enzymes that have an important function in olfaction; such as possibly to clear the pheromone from the acceptors after stimulation of the receptor membrane, as proposed by Kasang (1971).

The present report will primarily cover studies of pheromone catabolism in the cabbage looper moth, *T. ni*.

2. BIOCHEMICAL STUDIES OF PHEROMONE CATABOLISM

2.1. *Isolation of antennal esterases from chemosensory sensilla*

Esterases that degrade the *T. ni* pheromone, (*Z*)-7-dodecen-1-ol acetate, to the corresponding alcohol, (*Z*)-7-dodecen-1-ol, and acetic acid were isolated from chemosensory sensilla on the antennae (Mayer and Ferkovich, 1976). The structure of the olfactory sensilla allows the tips of the hairs to be broken off during their exposure to ultrasound (Figure 2). In a typical experiment, the antennae (125 pairs) were excised from laboratory reared 3-4-day-old adult moths into a 10 ml vial containing 1.0 ml of 0.5 M sucrose buffered with 0.05 M Tris-HCl, pH 7.5. They were then sonicated in an ice bath in a Ladd Model T-586® (82 kHz) ultrasonic cleaner at full power for 10-30 min. After sonication, the antennae were discarded and the sonicate was centrifuged at 6000 x *g* to remove scales and broken sensilla tips. 500 pairs of male antennae and 650 pairs of female antennae generally yielded about 0.38 mg and 0.2 mg of protein, respectively; 350 pairs of male or female legs yielded approximately 0.1 mg protein.

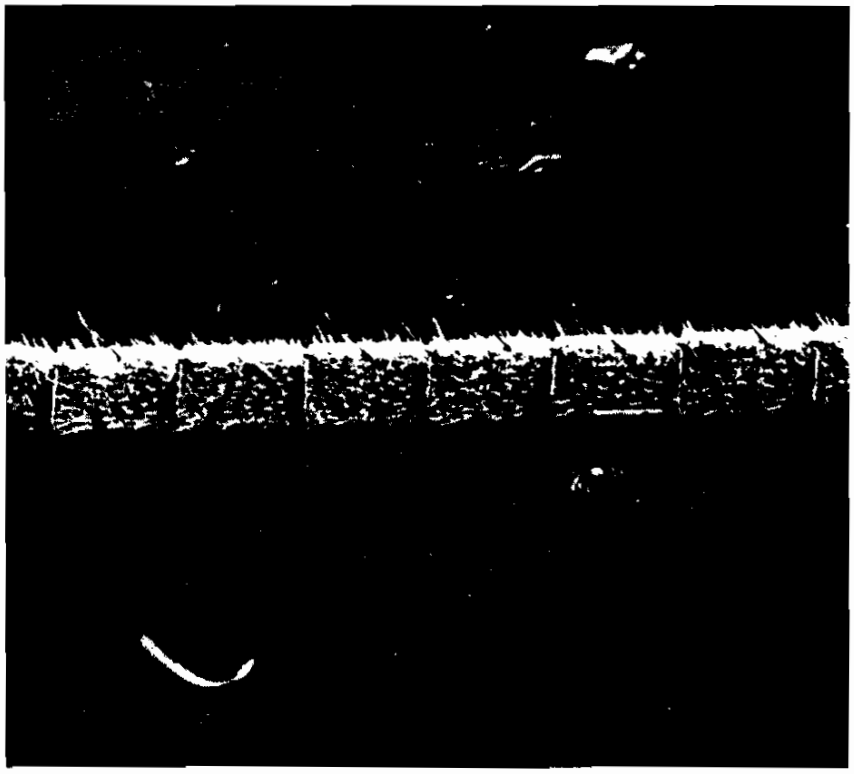

Figure 2. Demonstrates the initial purity of chemoreceptive elements obtained by sonication: A, typical flagellal segment of *T. ni* antennae prior to sonication, × 840 magnification; B, appearance of sensilla after 5-10 min sonication, × 1470; C, the antenna remains intact except for the fractured sensilla, × 98 (from Mayer and Ferkovich, 1976).

Ultracentrifugation of the sonicate at 100 000 × g for 2 h resulted in a soluble protein fraction and a microsomal pellet that was enriched in membrane vesicles, both of which have the enzymatic capacity to hydrolyze the pheromone (Mayer and Ferkovich, 1976).

Evidence that the pheromone-degrading esterases in the 100 000 × *g* supernatant were solubilized from the antennal membrane pellet was obtained from gel permeation and electrophoretic studies of the antennal sonicate. When the antennal sonicate was subjected to gel filtration before ultracentrifugation, two major absorption peaks were resolved (Figure 3). Assay of the fractions for pheromone-catabolizing esterases also revealed two peaks of activity. Treatment of the sonicate with Triton X-100® before gel filtration eliminated absorbance peak (a), and associated hydrolytic activity, near the void volume but left activity in the soluble enzyme peak (b). The apparent molecular weight (in Triton X-100) of the solubilized enzyme was 38 000. Likewise, treatment with phospholipase C, which disrupts the structure of membranes by hydrolyzing the phospholipid component, reduced the absorbance and enzyme activity of peak (a) but increased the enzymatic activity of peak (b). It, therefore, seems likely from these data that the detergent and phospholipase C either disrupted lipoprotein aggregates or solubilized membrane-bound enzyme.

Other evidence for the esterases in the 100 000 × g sonicate supernatant (Figure 4A)

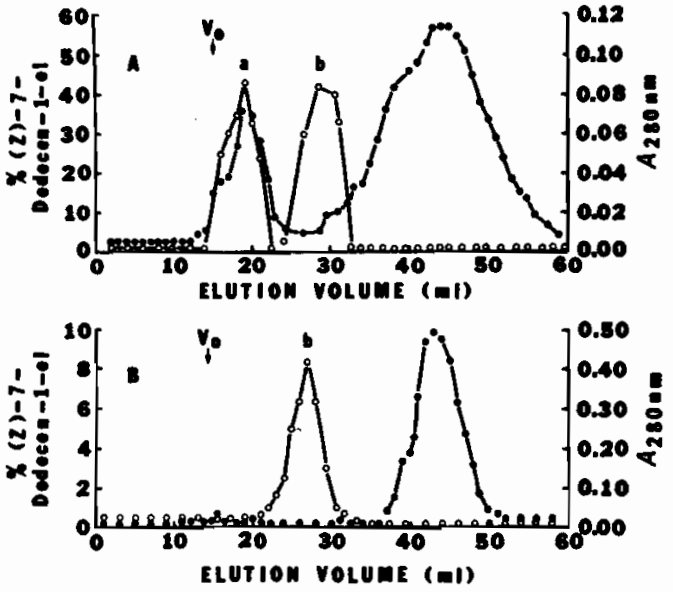

Figure 3. Effect of Triton X-100 on the elution pattern of male antennal sonicate through a column of Sephadex G-200 and enzymatic activity of individual fractions. A, lution pattern of antennal sonicate (0.2 mg protein) with 0.05 M Tris-HCl, pH 7.5, as eluant; B, elution pattern of antennal sonicate (1.9 mg protein) with 0.6% Triton X-100 in 0.05 M Tris-HCl, pH 7.5, as eluant (from Mayer and Ferkovich, 1976).

being removed from the nerve membrane pellet was obtained from the following experiment (Ferkovich et al., 1980). Whole antennae were homogenized and centrifuged at 20 000 × *g* for 30 min, thereby producing a homogenate-supernatant and pellet. Ultracentrifugation of the 20 000 × *g* supernatant at 100 000 × *g* for 2 h resulted in a soluble protein fraction and a microsomal pellet (Figure 4B). The microsomal pellet was resuspended in 0.5 ml sucrose buffer, sonicated with a probe-type sonicator and again centrifuged at 100 000 ×*g* for 2 h to produce a 100 000 ×*g* homogenate supernatant (Figure 4C). The resultant esterase pattern of this supernatant was the same as that of the 20 000 ×*g* supernatant. Furthermore, both of these esterase patterns were similar to that of the antennal sonicate supernatant (Figure 4A) which was obtained by fracturing the tips of the chemoreceptive sensilla during sonication of whole antennae. These results indicated that the esterases in the 100 000 ×*g* sonicate supernatant probably were solubilized from the nerve membranes. However, esterases have been detected in the cuticular pore canals and at the surface of the epicuticle after molting and are thought to function in wax synthesis (Locke, 1974). Therefore, it is possible that the esterases present in the membrane pellet were localized on the external surface of the antennae but were trapped within the membrane vesicles during homogenization. Mishra and Dev (1977) observed that non-specific esterases are associated with lysosomal granules situated at sites on peripheral and axonal membranes of neurons of sub-pharyngeal and segmental ganglia in leaches. Thus, it is also possible that the enzymes in *T. ni* were originally in soluble form or were associated with lysosomes within the antennae but were trapped in the membrane vesicles during isolation of the esterases.

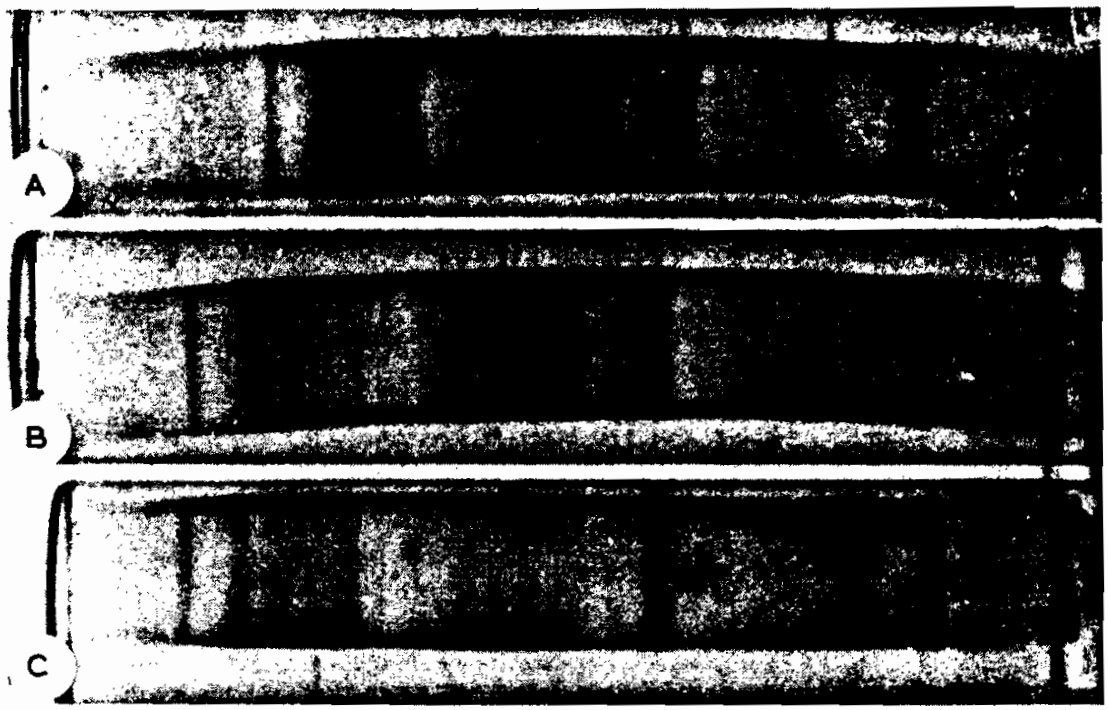

Figure 4. Demonstration that esterase bands observed in 100 000 × *g* supernatant of male antennal sonicate were solubilized from antennal nerve membranes. A, esterase pattern of 100 000 × *g* supernatant of antennal sonicate; B, esterase pattern of 20 000 × *g* supernatant of antennal homogenate; C, esterase pattern of 100 000 × *g* supernatant from resuspended 100 000 × *g* membrane pellet of antennal homogenate. Esterases (125 μg sample protein) were separated on 4% polyacrylamide gels and detected with 1-naphthyl acetate as substrate. Antennal sonicate in A was obtained by fracturing the tips of the chemoreceptive sensilla (see Figure 2). The membrane pellet in C was obtained by centrifuging the 20 000 × *g* supernatant in B at 100 000 × *g* for 2 h. The resultant membrane pellet was resuspended and centrifuged at 100 000 × *g* for 2 h to yield the homogenate-supernatant containing the solubilized esterases (from Ferkovich et al., 1980).

2.2. *Electrophoretic patterns of esterases from antennae and other tissues*

In earlier studies it was reported that antennae of male *T. ni* hydrolyze the pheromone at a rate higher than those of female *T. ni* (Mayer, 1975; Ferkovich et al., 1980). When the antennal esterases of both sexes were separated on polyacrylamide gels, no band unique to the male organ was detected that could be responsible for the difference in hydrolysis. However, it is highly probable that the electrophoretic gel system used was not capable of resolving minute differences in the esterase patterns.

When the esterase patterns of male antennae and eight other tissues were examined to obtain background information for subsequent studies on identification of possible pheromone-degrading enzymes, a larger number of disc gel electrophoretically resolved esterase bands was detected in the antennae than in the other tissues examined (Figure 5). 14 bands were detected in the antennae but only ten in the fat body and eight or less in the others. It was especially interesting to find that the nerve cord contained strikingly fewer bands than the antennae. These observations raised the question of whether any of

the extra bands in male antennae were responsible for the higher pheromone-degrading capacity of the antennae relative to that of other tissues (Ferkovich et al., 1972).

2.3. *Pheromone catabolism*

2.3.1. *Comparison of antennae with other tissues*

In view of the differences in esterase patterns of the antennae and various other tissues as resolved by disc gel electrophoresis, it was of interest to compare the conversion of radioactively labeled pheromone, (*Z*)-7-dodecen-1-ol acetate, to (*Z*)-7-dodecen-1-ol and acetic acid by the antennae, legs and two other tissues of *T. ni*. In earlier studies (Ferkovich et al., 1972; 1973), the antennae of *T. ni* hydrolyzed relatively more pheromone than did the legs and haemolymph. In this case, the pheromone was emulsified by ultrasonication, and metabolites were solvent-extracted and analyzed by gas-liquid chromatography (GLC). The greater catabolism of pheromone by antennae than by other tissues was further confirmed by using radioactively labeled pheromone (tritium-labeled in the acetate moiety) solubilized with fatty acid-free bovine serum albumin (Table 1). Metabolites of the pheromone were identified by thin-layer chromatography (TLC) (Ferkovich et al., 1980). The rate of hydrolysis for the male antennae was higher than that for the female antennae. Moreover, the antennae of both sexes had more pheromone-degrading activity than other tissues. However, results differed from those obtained from in vivo studies on pheromone catabolism in *T. ni* by Mayer (1975) in that several times more pheromone was hydrolyzed by the legs than by the antennae. The difference in hydrolysis was attributed to the greater surface area of the legs, but this interpretation implies that the enzymes are located on or near the cuticular surfaces of both organs. In fact, esterases have been located at the epicuticular surface where they are thought to function in wax synthesis after molting (Lock, 1967). However, in the case of the antennae, there is also evidence that suggests that pheromone-degrading esterases are membrane-bound, possibly in the dendritic nerve endings (Mayer and Ferkovich, 1976; Ferkovich et al., 1980).

Other pheromone-altering enzymes might operate in the antennae of *T. ni*. For example, an isomerase could convert the *cis* configuration of the natural pheromone molecule to the *trans*. Another possibility is the isomerization of the alcohol metabolite of the pheromone that has been shown to induce receptor potentials in the antenna (Mayer, 1975). Kassling (1974), however, stated that isomerization of bombykol into less active stereoisomers seemed improbable since the isomers also produced receptor potentials similar to bombykol and would have to undergo some type of inactivation. Mixed-function oxidases (MFO) may also be an important system for inactivation of the pheromone of *T. ni*. Because it has been shown that MFOs are involved in the biosynthesis of disparlure from an olefin in the gypsy moth, *Lymantria dispar* (Kasang et al., 1974b), it is also possible that the enzymes could complement the esterases in catabolizing the pheromone which is an olefin.

Figure 5. Esterase patterns of tissues of male *T. ni* separated on 4% polyacrylamide gels: A, male antennal sonicate; B, nerve cord; C, haemolymph; D, wing; E, flight muscle; F, body wall; G, fat body; H, malpighian tubules; I, mid- and hind-intestine. Antennal sonicate in A was obtained by fracturing the tips of the chemoreceptive sensilla (see Figure 2); the other tissues were homogenized. The antennal sonicate and supernatants were centrifuged at 20 000 × *g* for 30 min and the resultant supernatants (125 μg protein) were subjected to electrophoresis (from Ferkovich et al., 1980).

TABLE 1

COMPARISON OF PHEROMONE HYDROLYSIS BY SONICATE OF MALE ANTENNAE, AND BY HOMOGENATES OF LEGS AND TWO TISSUES

Values represent nmol pheromone hydrolyzed/min per μg protein and are means ± standard deviations of 10 replicates for antennae and five replicates for other sources. Antennal sonicate was obtained by fracturing tips of the chemosensory sensilla (Figure 2). Other sources were homogenized. The antennal sonicate and other tissues were centrifuged at 20 000 × *g* for 30 min and the resultant supernatants were used in the assay'. The assay solution consisted of $1.5 \cdot 10^{-5}$ M (*Z*)-7-dodecen-1-ol acetate (tritium-labeled in the acetate moiety, 804.4 mCi/mmol), bovine serum albumin (2 μg/μl) and 5 μg of sample protein in 100 μl 0.05 M Tris-HCl buffer (pH 8.0) at 22° C. The acetic acid and alcohol products were separated by TLC. Radioactivity was measured by liquid scintillation counting (from Ferkovich et al., 1980).

Enzyme source	Male	Female
Antennae	401 ± 57	294 ± 41
Legs	147 ± 19	76 ± 11
Fat body	56 ± 7	43 ± 5
Malpighian tubules	186 ± 42.6	155 ± 26

2.3.2. *Inhibition of antennal esterases*

The effects of five esterase inhibitors on the hydrolysis of radioactively labeled pheromone and 1-napthyl acetate, a substrate for general esterases, were compared. The results indicated that the esterases primarily responsible for catabolism of the pheromone were less sensitive to the inhibitors than those that hydrolyzed 1-naphthyl acetate (Table 2). The lower sensitivity of these pheromone-catabolizing esterases to the three inhibitors of the acetate suggests that part of the pheromone catabolism was produced by acetylesterases (Ferkovich et al., 1980).

Because results obtained with the inhibitors had indicated what types of esterases might be important in pheromone degradation, it was decided to identify the types of esterases (on polyacrylamide gels) and to determine whether any of the separated enzymes hydrolyzed the pheromone. The resolved esterase bands were classified on the basis of their sensitivity to three inhibitors, as described by Holmes and Masters (1967) for mammalian esterases. Male antennal esterases were separated on polyacrylamide gels, and the hydrolysis of pheromone upon incubation with a given 5 mm gel section containing esterases was measured (Figure 6). Two peaks of pheromone hydrolytic activity were observed. The bands with highest electrophoretic mobility (sections 8-9) had the highest pheromone degradative activity after a 1 min incubation with radioactively labeled pheromone. A carboxylesterase, two arylesterases and two fast-running acetylesterases were demonstrated in this region. The smaller pheromone hydrolytic peak (section 6) contained three enzymes classified as arylesterases. Of course, additional pheromone-degrading esterases may have been present in the sections but were not resolvable with

TABLE 2

HYDROLYSIS OF PHEROMONE AND 1-NAPHTHYL ACETATE BY 20 000 × *g* SUPERNATANT OF SONICATE: INFLUENCE OF VARIOUS INHIBITORS ON REACTION RATE

Pheromone hydrolysis was measured as described in Figure 1. The 1-naphthyl acetate assay consisted of 5 μg of sample protein in 1 ml of 0.1 M phosphate buffer, pH 6.5, containing $5 \cdot 10^{-4}$ M 1-naphthyl acetate. After incubation at 37°C for 30 min, 0.5 mg Fast Red TR salt was added and the absorbance was read at 625 nm. The percent inhibition is the mean percent reduction relative to control. Average of four replicates. The inhibitor was preincubated with the antennal sample (250 μg protein per 100 μl) for 20 min at 22°C before measurement of hydrolysis.

		% Inhibition			
		Pheromone		1-Naphthyl acetate	
Inhibitor concentration	(M)	Male	Female	Male	Female
Paraoxon	(10^{-6})	39.5	47.5	86.2	86.7
	(10^{-5})	57.0	62.8	99.3	95.3
	(10^{-4})	59.2	–	98.6	–
p-Mercuribenzoate	(10^{-4})	9.4	–	31.0	–
	(10^{-3})	33.4	44.8	92.7	87.2
Eserine sulfate	(10^{-5})	33.6	37.1	56.3	45.3
Ethopropazine hydrochloride	(10^{-4})	0	–	–	–
B.W. dibromide*	(10^{-5})	0	–	–	–

*1, 5-Bis (4-allyldimethyl ammonium phenyl) –pentane–3–one dibromide.

the gel systems used. Because the abdominal body wall contained four fast-running bands with relative mobilities (Figure 5A and F) that resembled the bands found in the antennae, similar quantities of antennal and body wall protein were separated on 4% gels, and pheromone hydrolysis was measured as in the earlier case with the antennae.

The resolved body wall esterases, like the antennal esterases, also exhibited the greatest activity in gel sections 6, 8 and 9; however, the rate of pheromone hydrolysis was higher for the antennal esterases than for the body wall esterases. For example, a 15 min incubation with enzymes from the antennae resolved in gel sections 8 and 9 hydrolyzed up to 96% of the pheromone; whereas, the enzymes in the same gel sections from the body wall preparation hydrolyzed less than 60%. These findings raise the question of whether the differences observed in pheromone-degradative activity between antennal and abdominal body-wall tissue are related to the function of the antennae in the olfactory process. Kinetic studies with purified enzymes from both tissues should aid in resolving this question.

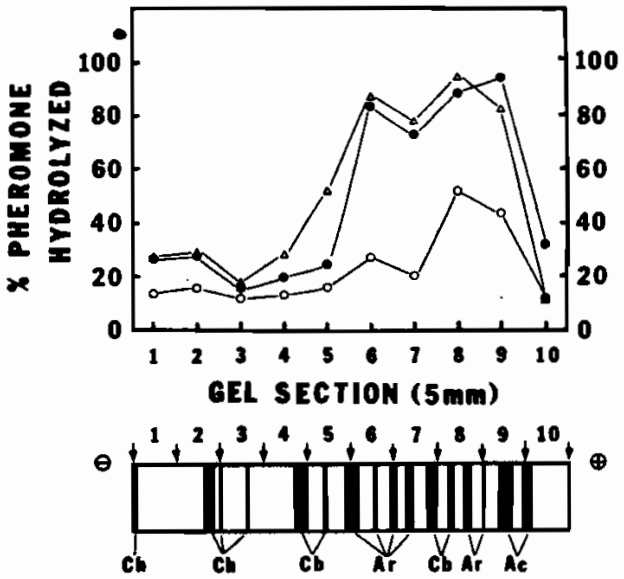

Figure 6. Hydrolysis of radioactively labeled pheromone by antennal esterases (125 μg protein) of male *T. ni* separated by electrophoresis on 4% polyacrylamide gels: ○, 1 min; ● 15 min; and △, 30 min incubation (37°C) of $1.5 \cdot 10^{-6}$ M pheromone with 5-mm gel sections in 0.05 M Tris-HCl, pH 8.0. Characterization of the esterases, detected in the gel sections with 1-naphthyl acetate, was based on the sensitivity of the enzymes to inhibitors as described by Holmes and Masters (1967) (from Ferkovich et al., 1980). Ar, arylesterase; Ac, acetylesterase; Cb, carboxylesterase; Ch, cholinesterase.

In view of earlier studies reported by Riddiford (1970) and Seabrook (1977), it was of interest to determine whether the pheromone-degrading enzyme(s) could be eluted from the antennae of *T. ni*. Riddiford reported binding of tritiated pheromone to proteins that were eluted from the silkworm antennae. Reception of the pheromone could be reversibly blocked by elution of the proteins, which were supposedly subjected to constant synthesis, and were thought to originate in the pore tubules of the chemosensory sensilla. However, it was not clear if the proteins were enzymes or receptor proteins, or if they were extracted from the cuticular surfaces rather than the pores of the hairs. Also, Seabrook observed that a surface layer on olfactory sensilla of the eastern spruce budworm moth could be digested away with proteinases. He proposed that the layer is extruded through the pores and onto the sensillum as a proteinaceous fluid. Therefore, studies similar to those of Riddiford (1970) were conducted to determine whether the enzyme could be eluted from the antennae of *T. ni*. Antennae of 72 live, 3-day-old adult male moths were held in Ringer's solution (80 μl/pair of antennae) for 12 h at 22° C. The eluant was dialyzed and concentrated (to 300 μl) with a -p Pro Di Mem® (10 000 mol. wt.-cutoff membrane) apparatus. It was then analyzed by disc gel electrophoresis for esterases and examined for pheromone-hydrolyzing activity as described by Ferkovich et al., 1980. The concentration of protein in the concentrated eluant was determined by the method of Lowry et al. (1951). For comparison of esterases in the eluant with those isolated from the chemosensory sensilla on the antenna, the 100 000 x *g* supernatant of a male antennal sonicate was also dialyzed and concentrated as described above, and the

pheromone-hydrolytic activity of the sample was examined. Incubation of 50 μl (10 μg protein) of the eluant and antennal sonicate each with $1.5 \cdot 10^{-5}$ M tritiated pheromone (as described in Table 1) resulted in a time-dependent hydrolysis of the pheromone. The percentage of total pheromone hydrolyzed after 1, 30, 60 and 120 min was 1.3, 2.2, 3.0 and 5.8 respectively, for the antennal eluant, and 37.6, 96.4, 97.4 and 98.2, respectively, for the antennal sonicate. The differences in pheromone-degradative activity between the two enzyme sources strongly suggested that the less active esterases in the eluant were washed off the cuticular surfaces of the antennae; whereas the more active esterases in the antennal sonicate originated from within the olfactory sensilla.

Separation of esterases in 200 μl of the eluant was carried out by electrophoresis on polyacrylamide gels as previously described (Ferkovich et al., 1980). Two faint bands were detected with relative mobilities of 0.40 and 0.77, thus resembling (in relative mobility) bands that were isolated from the antennae by sonication (Figure 5A). Apparently, the use of sonication to fracture the tips of the olfactory sensilla also extracts the esterases situated on the external surface of the antenna.

2.3.3. *Esterase changes during development*

Pheromone catabolism during late pupal and thoughout adult development of *T. ni* was measured by T. R. Taylor in our laboratory to determine if such hydrolytic activity could be related to responses to the pheromone as measured by the EAG method. If pheromone-degradative enzymes in the antenna are indeed involved in the olfactory process, then one might expect such enzymatic activity to increase with development of electrical responsiveness to the pheromone. Antennae were excised from pupae and were sonicated and centrifuged as described previously to isolate the esterases. As shown in Figure 7, hydrolysis was minimal until adult eclosion, when the rates for both males and females increased markedly. The enzymatic activity of the males peaked 2 days after eclosion and then began to decline slightly after 4 days. Payne et al. (1970) found that high EAG responses in antennae of males of *T. ni* were not generated until 0.25 day before eclosion. After emergence there was little variation in responsiveness to the pheromone with age. W. D. Seabrook (personal communication), however, found a maximal EAG response in *T. ni* males at 2-3 days after emergence. Schweitzer et al. (1976) reported that EAG responses to the pheromone in *Manduca sexta* (L.) begin 2 days prior to emergence, but increase dramatically on the day of eclosion. Thus, it appears that the insect is equipped to respond electrically to the pheromone on the day of emergence. In males of *T. ni* this electrical responsiveness is coincident with a striking increase in pheromone-degradative activity in the antenna 2-3 days after emergence. Incidentally, males of *T. ni* were reported to give the highest response to pheromone in behavioral assays 2-3 days after emergence (Shorey and Gaston, 1964).

The activity of the females was about 40% less than that of the males and peaked 3 days after eclosion. Grant (1970) found that females of *T. ni* produced EAG responses of lower magnitudes than those produced by males. This raises the question of whether the differences between male and female sensitivity are related to the lower enzyme activity measured in female antennae.

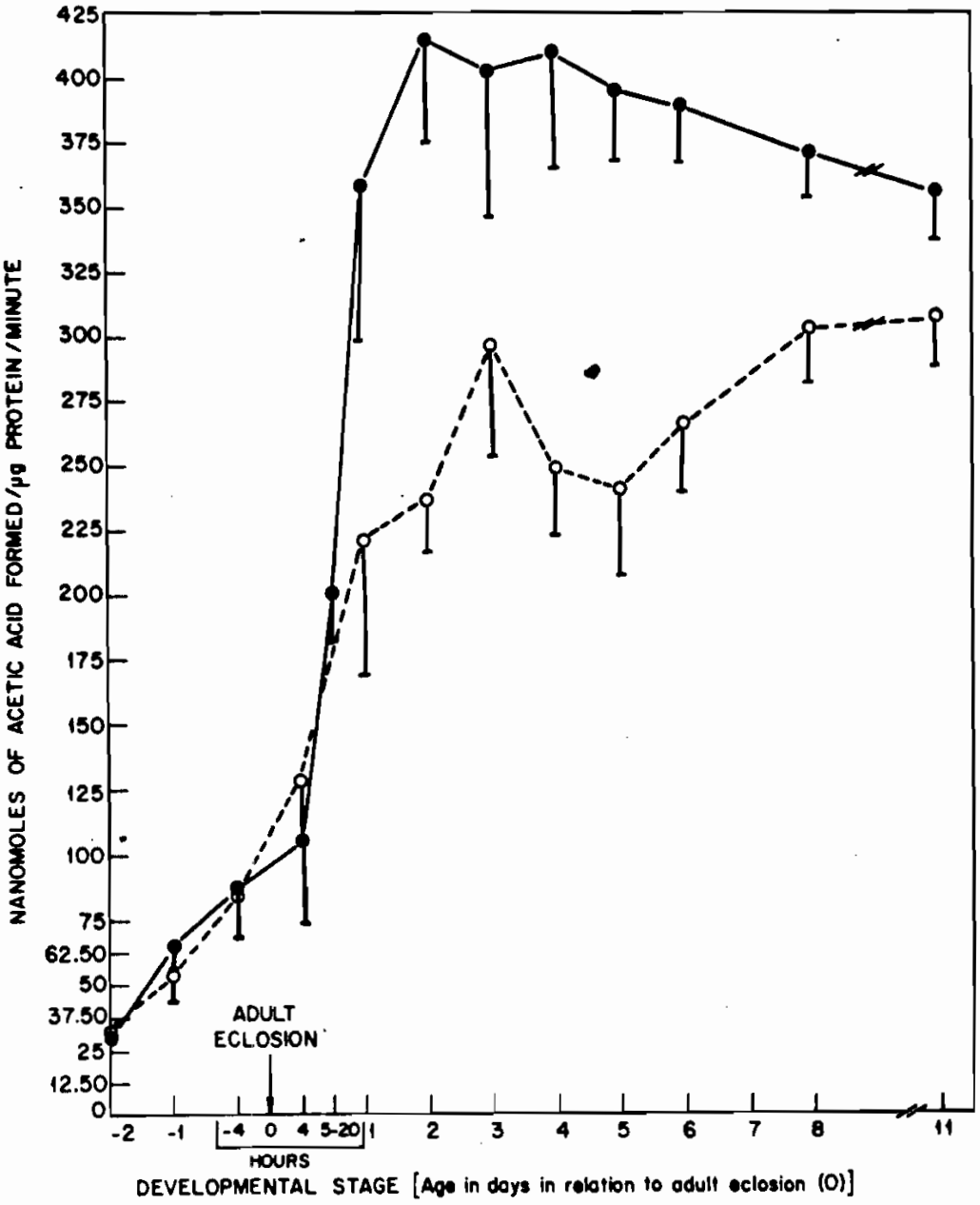

Figure 7. Hydrolysis of tritium-labeled *T. ni* pheromone upon incubation with antennal sonicates from male (•) and female (○) moths during different stages of development. See Table 1 for details of assay (courtesy of R. T. Taylor).

2.3.4. *Substrate specificity*

In vivo studies were conducted by Mayer (1975) to determine whether enzymatic specificity could be demonstrated with various isomers and analogs of the pheromone in *T. ni*. Antennae and legs of live moths were briefly dipped in a sonicated suspension of pheromone ($4.42 \cdot 10^{-3}$ M) in water and the remaining pheromone and its metabolites (alcohol and acid) were immediately extracted with rinses of solvent (diethyl ether) and

analyzed by GLC. The concentration of pheromone and isomers used was far above physiological levels, but this was necessary to quantitate metabolites of the chemicals and to compare the catabolism of each chemical under maximal velocity conditions. Male antennae degraded the pheromone at twice the rate that female antennae degraded it. In contrast, female antennae of *B. mori* degraded bombykol faster than did male antennae (Kasang and Kaissling, 1972).

Data in Table 3 show that specificity resulted if observations were confined to the first 4 s of the reaction when zero-order kinetics predominated. Hydrolysis was extremely rapid during the first 4 s of contact of pheromone with antennae and/or legs. Also, the pheromone was degraded more rapidly than other analogs and isomers, but this pattern of specificity was lost with longer incubations (over 1 min). These results differ from those reported for *B. mori* where bombykol and two analogs of the pheromone were all similarly degraded. However, in view of the results with *T. ni* it is possible that specificity with *B. mori* would have resulted had shorter incubation times (under 1 minute) been used as in *T. ni*. Clarification of this point must await purification of the enzymes and subsequent enzyme kinetic studies.

The interaction of the *T. ni* pheromone with antennal proteins in vitro was initially studied by using homogenized antennae of the cabbage looper (Ferkovich et al., 1973). Binding of the pheromone, (*Z*)-7-dodecen-1-ol acetate, was measured by ultraviolet-difference spectroscopy. The technique allowed distinction between enzymatic and non-enzymatic binding of the chemicals to the antennal proteins. The fact that the change associated with the pheromone was enzymatic was proven by measuring metabolites of the pheromone by GLC analysis. In addition, the pheromone was degraded more rapidly than the saturated analog. This is an indication of some structural specificity. Non-enzymatic binding was observed with the alcohol but not with the pheromone or saturated analog. This probably was because the more intense optical activity associated with enzymatic degradation of the pheromone overshadowed that of any non-enzymatic binding of the pheromone by the antennal proteins.

2.4. *Discussion*

The small amount of information available on enzymatic alteration of insect pheromones has dealt primarily with two species of Lepidoptera, the silkworm, *B. mori*, and the cabbage looper, *T. ni*. In the former case, catabolism of bombykol was considered to be a slow, unspecific process that serves to clean the stimulant from the olfactory hairs and other cuticular surfaces of the insect body after transduction (Kasang and Kaissling, 1972). In *T. ni* it appears that esterases capable of degrading the pheromone are located on the external surfaces of the antennae and possibly other body regions. Schneider (1970) proposed that enzymes may serve to prevent an accumulation of pheromone on the external surfaces of the insect which could otherwise desorb and interfere with chemical communication. In addition, some of the information on *T. ni* suggests that the conversion may be more closely related to the transduction process and may be somewhat analogous to the close association of acetylcholinesterase with acetylcholine receptors at excitable membranes (Rosenberry, 1976). Although precedent has been set for enzymes

TABLE 3

COMPARISON OF IN VIVO DEGRADATION OF PHEROMONE WITH SIX ISOMERS AND ANALOGS ON THE ANTENNA AND LEG OF MALE *T. NI* DURING 4-s INCUBATIONS

Antennae and legs of live moths (30 of each per test) were briefly dipped in a sonicated suspension of pheromone ($4.42 \cdot 10^{-3}$ M) in water; the remaining pheromone and its metabolites (alcohol and acetic acid) were extracted with rinses of solvent (diethyl ether) and analyzed by GLC (from Mayer, 1975). Ratio, isomer alcohol: pheromone alcohol.

Chemical	Uptake and degradation by antenna			Uptake and degradation by leg		
	Unchanged chemical recovered/antenna (μg)	Alcohol/antenna (ng)	Ratio	Unchanged chemical recovered/leg (μg)	Alcohol/leg (ng)	Ratio
(*Z*)-5-Dodecen-1-ol acetate	18.4	69.4	0.6	163.1	610.1	0.7
(*Z*)-7-Dodecen-1-ol acetate	25.6	118.1	1.0	40.3	865.8	1.0
(*Z*)-8-Dodecen-1-ol acetate	30.1	36.4	0.3	68.1	183.5	0.2
(*Z*)-9-Dodecen-1-ol acetate	27.3	<12.5	0.1	116.6	168.1	0.2
(*E*)-7-Dodecen-1-ol acetate	16.6	37.8	0.3	48.1	138.4	0.2
1-Dodecen-1-ol acetate	17.9	60.8	0.5	18.4	107.9	0.1
(*Z*)-7-Dodecen-1-ol butyrate	24.1	29.0	0.2	54.5	161.4	0.2

such as the α-glucosidases of contact sensilla on the labella of blow flies (Hansen, 1969; Kijima et al., 1973) possibly functioning as acceptor molecules for stimulants (sugars), the current data on *T. ni* neither support nor rule out the possibility that the enzymes are acceptors in the transduction mechanism.

The relevance of enzymatic alteration of pheromone to the olfactory process rests on the assumption that the enzymes are associated with the chemosensory sensilla. The following evidence supports this assumption. Over 90% of the sensilla on the antennae of *T. ni* are thin-walled olfactory hairs (sensilla trichoidea); and of these sensilla, approximately 50% have cells that respond to the pheromone (M. S. Mayer, personal communication). Therefore, it is assumed that at least some of the esterases released upon fracturing the tips of the sensilla by sonication originate from the primary receptor cells for the pheromone. Although the exact location of the pheromone-degrading enzymes within the sensillum is not known and awaits further investigation, evidence strongly suggests that the enzymes are bound to membranes isolated in the microsomal pellet. Also, the preliminary results of several membrane-specific marker enzyme assays revealed a predominance of acetylcholinesterase and $(Na^+ + K^+)$-ATPase activities (Michelot et al., 1978) in the pellet, which indicates the presence of plasma membranes. Therefore, it is possible that the pheromone-degrading enzymes are associated with the dendritic nerve endings of the receptor neurons. Other data that support the assumption that the enzymatic conversion is an event closely related to olfactory perception in *T. ni* are those on the enzyme specificity observed in the in vivo studies, and the appearance of pheromone degradative activity in the antennae after pupal ecdysis when the moth begins to respond to the attractant.

Pheromone degradation is an important biological function in insects and should receive more attention. It may also be possible to utilize this information in the development of new methods to control insect pests. For example, the enzyme may be a vulnerable point of attack in the insect's communication system. Possibly inhibition of the pheromone-converting enzymes by chemical means might interfere with perception of the pheromone. Further effort should also be made to determine how pheromone is degraded in other species. This is especially important in insects of orders other than Lepidoptera and insects with pheromones of varied structure.

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